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RESEARCH ARTICLE

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Description and DNA barcoding of three new species of *Leohumicola* from South Africa and the United States

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Key words

aleurioconidium
chlamydospore
cytochrome oxidase subunit I (*Cox1*)
internal transcribed spacer (ITS)
Leotiomycetes

Abstract Three new species of *Leohumicola* (anamorphic Leotiomycetes) are described using morphological characters and phylogenetic analyses of DNA barcodes. *Leohumicola levissima* and *L. atra* were isolated from soils collected after forest fires in Crater Lake National Park, United States. *Leohumicola incrustata* was isolated from burned fynbos from the Cape of Good Hope Nature Reserve, South Africa. The three species exhibit characteristic *Leohumicola* morphology but are morphologically distinct based on conidial characters. Two DNA barcode regions, the Internal Transcribed Spacer (ITS) nuclear rDNA region and the cytochrome oxidase subunit I (*Cox1*) mitochondrial gene, were sequenced. Single-gene parsimony, dual-gene parsimony and dual-gene Bayesian inference phylogenetic analyses support *L. levissima*, *L. atra*, *L. incrustata* as distinct phylogenetic species. Both ITS and *Cox1* barcodes are effective for the molecular identification of *Leohumicola* species.

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INTRODUCTION

The hyphomycete genus *Leohumicola* was described for four species (*L. verrucosa*, *L. minima*, *L. terminalis*, and *L. lenta*) by Hambleton et al. (2005). *Leohumicola* species produce two-celled aleurioconidia, with a round to ellipsoidal, dark-brown terminal cell with slightly thickened walls, and a basal cell that is either cupulate or cylindrical, and hyaline to pale brown. Globose to ellipsoidal, intercalary or terminal chlamydospores are produced by all known species. Conidium ontogeny is usually monoblastic, with sympodial extension of the conidiogenous cells sometimes occurring. Secession is rhexolytic, with the remnants of the empty basal cell remaining attached to the terminal cell. Most *Leohumicola* strains grow slowly and sporulate sparsely or not at all; they must be grown on various media to stimulate conidial production.

Hambleton et al. (2005) noted that many internal transcribed spacer (ITS) sequences of unidentified soil or root-associated fungi in GenBank belonged to the *Leohumicola* clade, but did not correspond with the species they described. Several of the known species of *Leohumicola* were associated with burnt ecosystems, especially commercial blueberry cultivation, and were isolated by heat treatment of soil suspensions or from surface-sterilised roots of ericaceous host plants. This association with the plant family Ericaceae led us to obtain soil samples from a burned area of fynbos in the Cape Floristic Region (Cape of Good Hope Nature Reserve, South Africa), a hotspot of biodiversity for this plant family (Cowling & Richardson 1995). We also obtained soil from a part of Crater Lake National Park, United States, recently affected by forest fires.

The original study of *Leohumicola* supplemented morphological information with phylogenetic analysis of the ITS. Here, we add analyses of cytochrome oxidase subunit I (*Cox1*) mitochondrial gene sequences. The ITS is a widely accepted DNA marker for identifying fungi. *Cox1* is the DNA barcode gene that has been tested most extensively in the animal kingdom, with a 648-bp region in the 5' end usually providing species-level resolution (e.g. Ward et al. 2005, Hajibabaei et al. 2006). The *Cox1* barcode region was somewhat effective in identifying species of *Penicillium* (Seifert et al. 2007) but has otherwise been little explored in fungi. This study assesses the utility of both ITS and *Cox1* as DNA barcodes for the identification of *Leohumicola* species.

MATERIALS AND METHODS

Isolation, observation and preservation of cultures

Soil samples from South Africa were collected at Olifantsbos, Cape of Good Hope Nature Reserve, with the kind assistance of Dr Karin Jacobs and her students from the Department of Microbiology, University of Stellenbosch. The sampled region was a fynbos with abundant *Erica* and *Protea* species, which had been burned one or two years previously. Samples of surface and rhizosphere soil were collected in sterile 15 mL BD Falcon conical bottom tubes and mailed to Ottawa. Soil samples from Crater Lake National Park, Montana, USA, were collected by Matt Trappe in a region with visible signs of forest fire damage. Approximately 50 g samples were collected into zip lock bags and mailed to Ottawa. All samples were kept at room temperature until processing.

Fungi were isolated using a method modified from Jackson et al. (1995). After using a layer of cheesecloth to remove large particles from the soil sample, 2–4 mL of fine dry soil were placed into 50 mL BD Falcon conical bottom tubes. Sterile 0.1 % (w/v) peptone broth was added to the 25 mL mark, then the tube was vortexed for 30 s. The tube was submerged in a 75 °C water bath until the suspension reached this temperature (~ 15 min),

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Table 1 Source of isolates and GenBank accession numbers of 52 strains used in this study.

		Source				GenBank accession	
Species	Culture	Country	State/Province	Region	Associated plants or substrate	ITS	*Cox1*
Leohumicola atra	DAOM239515[T]	United States	Oregon	Crater Lake National Park	*Goodyera oblongifolia*	EU678386	EU678425
	DAOM239535	United States	Oregon	Crater Lake National Park	*Goodyera oblongifolia*	EU678411	EU678450
Leohumicola incrustata	DAOM239498[T]	South Africa	Cape Province	Cape of Good Hope N.R.	*Erica*	EU678398	EU678437
	DAOM239500	South Africa	Cape Province	Cape of Good Hope N.R.	*Erica*	EU678409	EU678448
	DAOM239501	South Africa	Cape Province	Cape of Good Hope N.R.	*Erica*	EU678399	EU678438
	DAOM239502	South Africa	Cape Province	Cape of Good Hope N.R.	*Erica*	EU678404	EU678443
	DAOM239503	South Africa	Cape Province	Cape of Good Hope N.R.	*Erica*	EU678406	EU678445
	DAOM239504	South Africa	Cape Province	Cape of Good Hope N.R.	*Erica*	EU678402	EU678441
	DAOM239505	South Africa	Cape Province	Cape of Good Hope N.R.	*Erica*	EU678403	EU678442
	DAOM239517	South Africa	Cape Province	Cape of Good Hope N.R.	*Protea* tree	EU678397	EU678436
	HNLHM91	South Africa	Cape Province	Cape of Good Hope N.R.	*Erica*	EU678400	EU678439
	HNLHM92	South Africa	Cape Province	Cape of Good Hope N.R.	*Erica*	EU678401	EU678440
	HNLHM101	South Africa	Cape Province	Cape of Good Hope N.R.	*Erica*	EU678405	EU678444
	HNLHM103	South Africa	Cape Province	Cape of Good Hope N.R.	*Erica*	EU678407	EU678446
	HNLHM107	South Africa	Cape Province	Cape of Good Hope N.R.	*Erica*	EU678408	EU678447
Leohumicola lenta	DAOM231149[T]*	Canada	Manitoba		tallgrass prairie	AY706328	EU678465
Leohumicola levissima	DAOM239506[T]	United States	Oregon	Crater Lake National Park	*Abies concolor*	EU678382	EU678421
	DAOM239507	United States	Oregon	Crater Lake National Park	*Abies concolor*	EU678384	EU678423
	DAOM239508	United States	Oregon	Crater Lake National Park	*Abies concolor*	EU678385	EU678424
	DAOM239509	United States	Oregon	Crater Lake National Park	*Abies concolor*	EU678389	EU678428
	DAOM239510	United States	Oregon	Crater Lake National Park	*Abies concolor*	EU678394	EU678433
	DAOM239511	United States	Oregon	Crater Lake National Park	*Goodyera oblongifolia*	EU678396	EU678435
	DAOM239512	United States	Oregon	Crater Lake National Park	*Ceanothus velatinus*	EU678388	EU678427
	DAOM239513	United States	Oregon	Crater Lake National Park	*Abies concolor*	EU678395	EU678434
	DAOM239514	United States	Oregon	Crater Lake National Park	*Abies concolor*	EU678392	EU678431
	HNLHM2	United States	Oregon	Crater Lake National Park	*Abies concolor*	EU678380	EU678419
	HNLHM2B	United States	Oregon	Crater Lake National Park	*Abies concolor*	EU678381	EU678420
	HNLHM4	United States	Oregon	Crater Lake National Park	*Abies concolor*	EU678383	EU678422
	HNLHM21	United States	Oregon	Crater Lake National Park	*Ceanothus velatinus*	EU678387	EU678426
	HNLHM41	United States	Oregon	Crater Lake National Park	*Abies concolor*	EU678390	EU678429
	HNLHM42	United States	Oregon	Crater Lake National Park	*Abies concolor*	EU678391	EU678430
	HNLHM47	United States	Oregon	Crater Lake National Park	*Abies concolor*	EU678393	EU678432
	HNLHM144	United States	Oregon	Crater Lake National Park	*Goodyera oblongifolia*	EU678412	EU678451
	HNLHM145	United States	Oregon	Crater Lake National Park	*Goodyera oblongifolia*	EU678413	EU678452
	HNLHM152	United States	Oregon	Crater Lake National Park	*Goodyera oblongifolia*	EU678414	EU678453
Leohumicola minima	DAOM232587[T]*	Chile		Valdivia	volcanic ash	AY706329	EU678466
Leohumicola sp.	DAOM230084*	Australia	N.S.W.		*Eucalyptus*	AY706331	EU678455
	DAOM231148*	Canada	Manitoba		tallgrass prairie	AY706330	EU678461
	DAOM239499	South Africa	Cape Province	Cape of Good Hope N.R.	*Lobelia*	EU678417	EU678469
	DAOM239516	Canada	Ontario	Stittsville	forest soil	EU678416	EU678468
Leohumicola terminalis	DAOM231145[T]*	Canada	Nova Scotia		*Acer saccharum*	AY706327	EU678464
Leohumicola verrucosa	DAOM226889[T]*	Canada	Nova Scotia		blueberries	AY706320	EU678462
	DAOM231141*	Canada	Alberta		*Pinus banksiana, Populus tremuloides*	AY706321	EU678457
	DAOM231142*	Canada	Nova Scotia		blueberries	AY706322	EU678458
	DAOM231143*	Canada	Nova Scotia		*Pinus* spp.	AY706323	EU678463
	DAOM231144*	Canada	Nova Scotia		*Pinus strobes*	AY706324	EU678459
	DAOM231147*	Canada	Alberta		*Pinus* forest	AY706325	EU678460
	DAOM230085*	Puerto Rico			not recorded	AY706326	EU678456
	DAOM239497	Canada	Ontario	Stittsville	forest soil	EU678379	EU678418
	DAOM239536	Canada	Ontario	Stittsville	forest soil	EU678415	EU678454
	HNLHM117	Canada	Ontario	Stittsville	forest soil	EU678410	EU678449
Myxotrichium deflexum	UAMH6365**	Canada	Ontario	Toronto	soil	AF062814	EU678467

* *Leohumicola* strains previously studied by Hambleton et al. (2005). The ITS GenBank accession numbers from Hambleton et al.'s study are listed here for completeness. *Cox1* DNA barcodes of *Leohumicola* species described by Hambleton et al. (2005) were generated in this study.

** *Myxotrichium deflexum* strain UAMH6365 is from Hambleton et al. (1998).

Abbreviations: DAOM = Canadian Collection of Fungal Cultures, Ottawa, Canada; HNLHM = Seifert Lab *Leohumicola* Collection, T = ex-type strain; N.R. = Nature Reserve; N.S.W. = New South Wales.

then incubated for a further 30 min with manual shaking every 5 min to ensure even temperature distribution. The tube was cooled at room temperature for 30 min. A well homogenised 1 mL aliquot of the suspension was mixed with 100 mL of molten and cooled (50 °C) half-strength potato-dextrose agar (PDA (Difco), BD, Sparks, Maryland, USA) with 40 mg/L chloramphenicol to inhibit bacterial growth. The mixture was dispensed in polystyrene Petri dishes (~ 20 mL per dish) and then left to solidify. Petri dishes were sealed with Parafilm and incubated upright at 25 °C under ambient light conditions.

Isolation plates were checked for *Leohumicola* colonies every 1–2 d for 2 wk. Petri dishes with no visible growth after 2 wk were incubated for up to 3 mo before disposal. *Leohumicola* colonies were recognised by their slow growth combined with the release of yellow pigments into the medium. Putative *Leohumicola* colonies were transferred to new full-strength PDA plates, then incubated as above for 3 wk at room temperature (22–25 °C) before performing morphological studies and DNA extraction.

For morphological studies, suspected *Leohumicola* colonies were grown on oatmeal agar (OA, Samson et al. 2004), corn meal agar with dextrose (CMA (Difco), BD, Sparks, Maryland, USA) and PDA. To induce sporulation, PDA was inoculated with a suspension of macerated mycelium as described by Hambleton et al. (2005). Cultures were checked for aleurioconidia and chlamydospores monthly. Cultures that did not sporulate after 3 mo were transferred to potato-carrot agar (PCA, Samson et al. 2004), incubated at 25 °C under ambient light conditions for 2 mo, and then rechecked for conidia. Measurements and photographs were taken from material mounted in 85 % lactic acid using an Evolution MP digital microscope camera on an Olympus BX50 compound microscope with differential interference contrast (DIC) optics and captured using ImagePro 6.0 (Media Cybernetics, Bethesda, Maryland, USA). Colony photographs were taken after 2 wk and 2 mo. Some microphotographs and colony photographs were digitally retouched using Adobe Photoshop CS2 for aesthetic reasons, to reduce background clutter and to remove unwanted reflections, as noted in the figure legends. Colony colours were assessed using Kornerup & Wanscher (1978).

Twenty-three strains selected to represent the genetic diversity of the isolated species were deposited in the Canadian Collection of Fungal Cultures (DAOM), Ottawa, Canada (Table 1). Additional strains are deposited in the Seifert Lab collection at Agriculture and Agri-Food Canada.

DNA extraction, PCR, sequencing and sequence editing

DNA extractions were performed using UltraClean Microbial DNA Isolation Kits (MO BIO Laboratories Inc., Carlsbad, California, USA) from mycelia scraped from PDA colonies using a sterile scalpel. DNA concentration and quality were determined by Nanodrop ND-1000 spectrometer (Thermo Scientific, Wilmington, Delaware, USA) and preparations were diluted to 1–5 ng/µL of DNA template.

The ITS and *Cox1* regions were amplified and sequenced using the primers ITS5 and ITS4 (White et al. 1990) and newly designed primers for the *Cox1* of the *Pezizomycotina*,

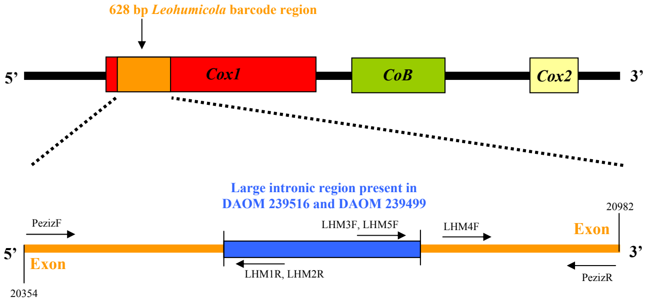


Fig. 1 Primer binding sites for the 628 bp *Leohumicola Cox1* barcode region. Reference positions based on the mitochondrial genomic sequence of *Saccharomyces cerevisiae*.

PezizF (5'-TCAGGRTTAYTAGGWACAGCATTT-3') and PezizR (5'-ACCTCAGGRTGYCCGAAGAAT-3') (S. Gilmore, pers. comm.). Primers ITS1, ITS2, and ITS3 (White et al. 1990) were sometimes used as internal sequencing primers when the DNA sequence quality obtained from ITS5 and ITS4 was inadequate. Primer binding sites for *Cox1* are illustrated in Fig. 1.

For the PCR master mix, 0.1 mM dNTP's, 0.08 µM forward primer, 0.08 µM reverse primer, 1X Titanium Taq buffer (Clontech, Mountain View, California, USA), 0.5X Titanium Taq enzyme (Clontech, Mountain View, California, USA), and 1.00 µL of DNA template (1–5 ng/µL) were mixed in sterile HPLC water totalling 10 µL per reaction. The PCR reaction was run in a Mastercycler epgradient S thermal cycler (Eppendorf, Mississauga, Ontario, Canada). The following profile was used to amplify ITS: 95 °C for 3 min (initial denaturation), then 40 cycles at 95 °C for 45 s (denaturation), 60 °C for 45 s (annealing), 72 °C for 1.5 min (extension), then 72 °C for 8 min (final extension). The following parameters were used to amplify *Cox1*: 95 °C for 3 min (initial denaturation), then 40 cycles at 95 °C for 1 min (denaturation), 51 °C for 1 min (annealing), 72 °C for 1.5 min (extension), then 72 °C for 8 min (final extension).

For sufficient amplification of *Cox1* for *Leohumicola* sp. DAOM 239516, a touchdown PCR was performed. The *Cox1* touchdown profile was the same as the profile described above except that the annealing temperature started at 54 °C (5 cycles), then changed to 51 °C (5 cycles), then to 49 °C (5 cycles), then finally to 46 °C (35 cycles), for a total of 50 cycles. For sufficient *Cox1* amplification from *Myxotrichum deflexum* UAMH 6365, a step-up PCR was performed. The *Cox1* step-up profile differed with the annealing temperature initially at 46 °C (10 cycles), then 49 °C (10 cycles), then finally 51 °C (30 cycles), for a total of 50 cycles. PCR products were separated by electrophoresis on a 1 % agarose gel, stained with ethidium bromide and visualised under UV light.

Both forward and reverse strands were sequenced using Big Dye Terminator (Applied Biosystems, Foster City, California, USA) in 10 µL reactions with the same protocol described by de Cock & Levesque (2004). The following profile was used for the sequencing reaction of ITS: 95 °C for 3 min, then for 40 cycles at 95 °C for 30 s, 50 °C for 15 s, 60 °C for 2 min. For *Cox1*, the sequencing reaction profile was 95 °C for 3 min, then 40 cycles at 95 °C for 30 s, 51 °C for 15 s, 60 °C for 4 min.

Table 2 Comparative summary of colony and microscopic characters of all currently known *Leohumicola* species.

Species	Colony characters after 2 wk				Aleurioconidia characteristics (PDA and CMA)			
	PDA			OA				
	diam (mm)	mycelial colour	soluble pigments	diam (mm)	position	terminal cell (µm)	wall	basal cell (µm)
Leohumicola atra	10	olive	greyish red	13–15	lateral and terminal	4.5–5.5 × 4.0–5.5	smooth dark brown	2.5–4.5 × 2.5–3.5
Leohumicola incrustata	12–18	pastel yellow, greyish yellow, grey	olive-brown, dark yellow	15–20	lateral and terminal	4.0–5.5 × 4.0–5.0	verrucose and slimy	2.5–4.5 × 2.0–3.0
*Leohumicola lenta**	1	olive	minimal	3–4	lateral and terminal	7.0–10 × 6.5–8.5	smooth	4.0–11.0 × 2.0–5.0
Leohumicola levissima	15–20	olive, grey	minimal olive	12–17	lateral and terminal	4.5–6.0 × 4.0–5.5	smooth	1.5–4.0 × 2.5–3.5
*Leohumicola minima**	20–22	grey, olive grey, brownish grey	olive-grey, brownish grey	18	lateral and terminal	4.5–8.0 × 3.5–5.0	smooth or partly verrucose	2.0–5.5 × 2.0–3.0
*Leohumicola terminalis**	10	greyish yellow	olive	11–12	terminal only	5.0–7.5 × 5.0–8.5	slightly rough	3.0–8.5 × 2.5–4.5
*Leohumicola verrucosa**	12–18	greyish yellow, olive yellow, grey	red, reddish brown, olive brown	15–19	lateral and terminal	4.0–5.5 × 4.0–5.5	verrucose (usually)	2.0–4.5 × 2.5–3.0

* *Leohumicola* species previously studied by Hambleton et al. (2005).

Contigs were assembled and edited using SeqMan II v7.0 from DNA Star (www.dnastar.com/). To confirm that the newly designed PCR primers amplified the expected gene, BLAST analyses were performed with our putative *Cox1* sequences to verify that they were homologous with other fungal *Cox1* sequences. All sequences are deposited in GenBank and the Barcode of Life Database (BOLD, www.barcodinglife.org) (see Table 1).

Sequence alignment and phylogenetic analyses

Internal Transcribed Spacer (ITS) and *Cox1* sequences were aligned using MAFFT v6 (Katoh et al. 2005). A few minor adjustments were made to the ITS alignment using Se-Al v. 1.0 (Rambaut 1996). No manual adjustments were required for the *Cox1* alignment, which had no indels. Alignments are deposited in TreeBASE (www.treebase.org/treebase/), study accession no. S2134. To test whether the ITS and *Cox1* data sets contained congruent phylogenetic signals and could be combined for analysis, a partition homogeneity test (Farris et al. 1994) was performed using PAUP 4.0 (Swofford 2002) using a heuristic search with 1 000 replicates, TBR branch swapping, unordered and unweighted characters and gaps treated as missing.

Parsimony analyses of ITS alone (Fig. 4a), *Cox1* alone (Fig. 4b), and for both genes combined (Fig. 4c), were performed using heuristic searches in PAUP 4.0 (Swofford 2002), with uninformative characters excluded. Bootstrap analyses (1 000 replicates) were undertaken using full heuristic searches for the two single-gene parsimony analyses, and using fast-stepwise addition for the dual-gene analysis. For all parsimony analyses, parsimony tree scores were calculated and the 70 % consensus tree was computed.

For Bayesian analysis, MrModeltest v. 2.2.6 (Nylander 2004) was used to select the most appropriate models of sequence evolution for data sets that contained *Leohumicola* species only, according to the Akaike information criterion (AIC) (Akaike 1974). The HKY+I model (Hasegawa et al. 1985) and the K80+G (Kimura 1980) model were selected for *Cox1* and ITS, respectively. Bayesian inference was performed with MrBayes v. 3.1.2 (Ronquist & Huelsenbeck 2003) using the dual-gene data set with two designated partitions (ITS and *Cox1*), the appropriate model of sequence of evolution applied to each partition, and *L. lenta* DAOM 231149 set as outgroup (see below). Two independent MCMC runs were performed simultaneously. Each MCMC ran for 2.0×10^6 generations, sampling every 100 generations, for a total of 20 001 trees. Acceptable convergence was attained after 1.0×10^6 generations, and the first 10 000 trees were discarded as burn-in. The 10 001 trees from each independent MCMC (total 20 002 trees) were combined into one consensus tree with 50 % majority rule consensus (Fig. 4d).

Myxotrichum deflexum was initially chosen as an outgroup to root all analyses, based on its position as near neighbour to the *Leohumicola* clade in the 18S analyses by Hambleton et al. (2005). We could not obtain satisfactory sequences for the *Cox1* of two other potential outgroups, *Myxotrichum arcticum* UAMH 9243 and *Scytalidium lignicola* DAOM 231160. Therefore, *M. deflexum* was used as the outgroup for the single-gene parsimony analyses. In the Bayesian analysis, the branch

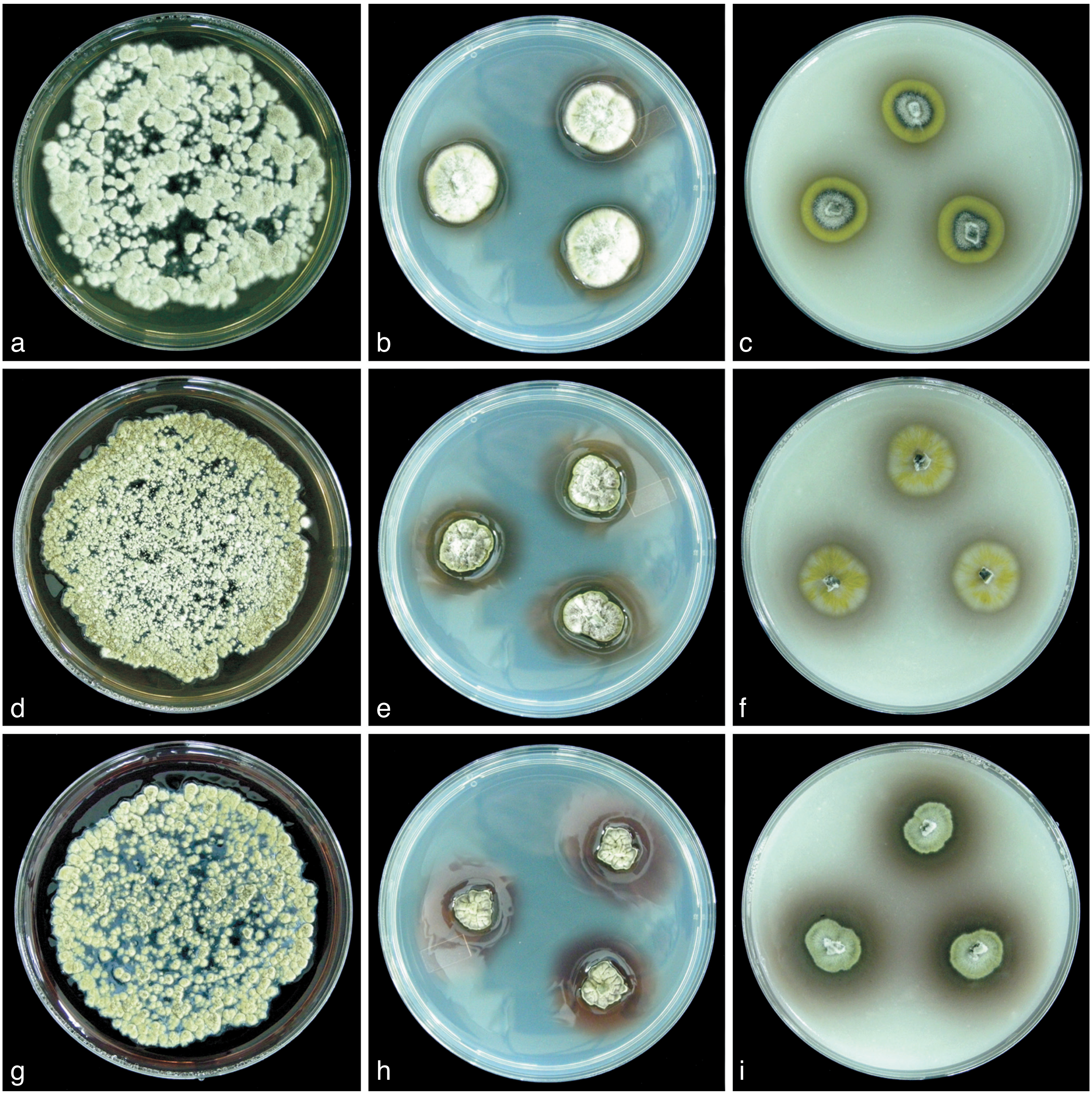


Fig. 2 Three *Leohumicola* species growing from macerated inocula on PDA (left column), as three point inocula on PDA (middle column) and OA (right column), after 2 wk at room temperature. a–c. *L. levissima* HNLHM2; d–f. *L. incrustata* DAOM 239498; g–i. *L. atra* DAOM 239515. Reflections on the shiny agar surface from the middle column pictures were removed digitally. — Petri dish diam = 9 cm.

connecting *M. deflexum* to the ingroup was too long, obscuring the phylogenetic structure of the ingroup. Therefore, *L. lenta* was used to root the tree for the Bayesian and dual-gene parsimony analyses because of its basal position in the single-gene parsimony analyses.

Two *Leohumicola* isolates (DAOM 239499, 239516) had an intron in the *Cox1* region. Internal sequencing primers LHM1R (5'-GGCGTTCTTAGTTCTCCATTTAGT-3'), LHM5F (5'-TTAAGTGGGGTACAAAGTCA-3') and LHM4F (5'-GGTATAGAAAATGGAGCAGGTA-3') were designed and used to sequence the poorly resolved region at the ends of the exonic region for *Leohumicola* sp. DAOM 239499. Similarly internal sequencing primers LHM2R (5'-GGCGTTCTTAGTTTTCCATT-3'), LHM3F (5'-CCGCCTAGTTTATTATTATTTTTA-3') and LHM4F were used for the intron of *Leohumicola* sp. DAOM 239516.

RESULTS

By heat treating soil, three distinct species exhibiting characteristic *Leohumicola* morphology were isolated. On PDA, colonies

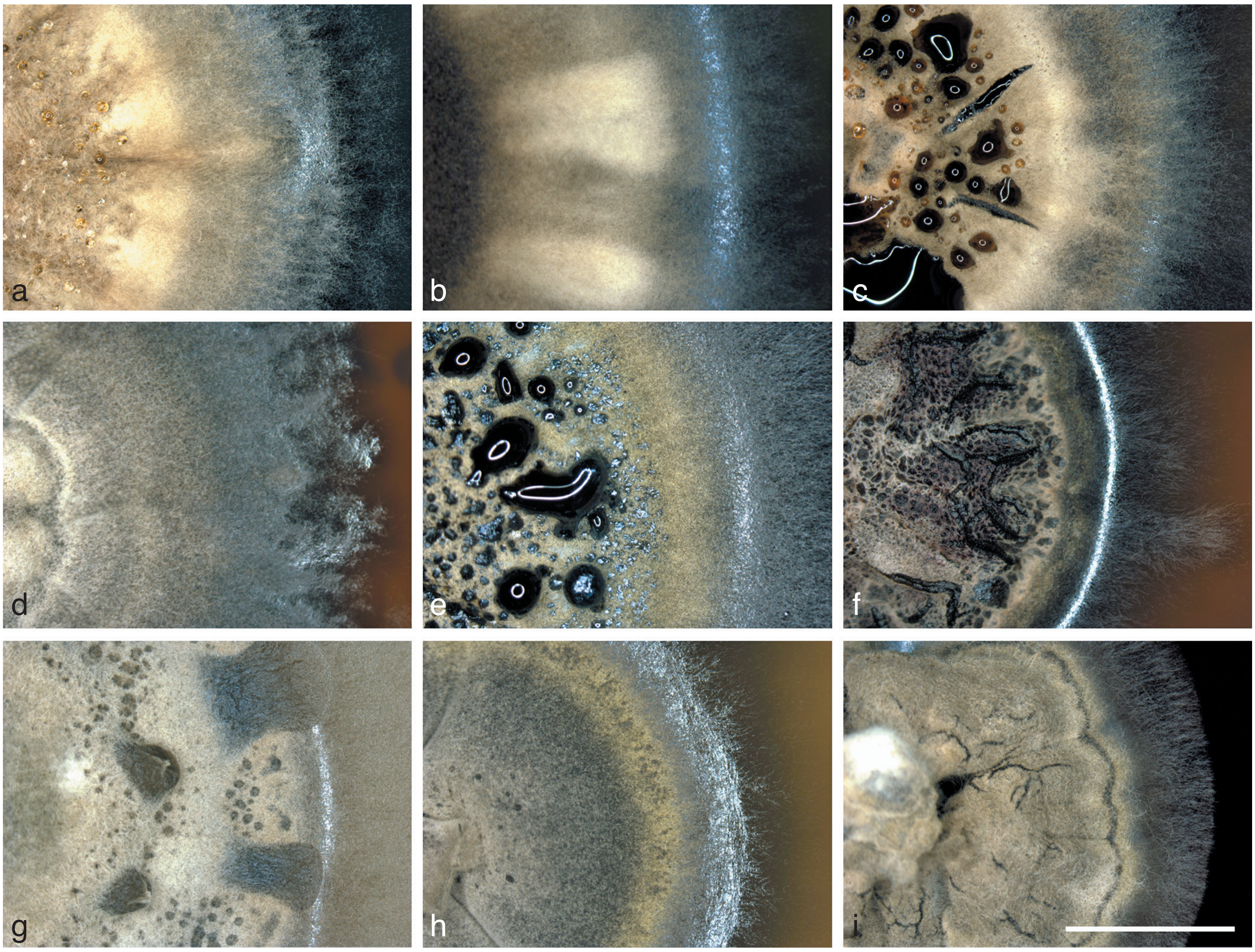


Fig. 3 *Leohumicola* colonies on PDA after 2 mo incubation. a. *L. levissima* HNLHM2B; b. *L. levissima* DAOM 239511; c. *L. levissima* DAOM 239512 with dark brown exudates; d. *L. atra* DAOM 239515; e. *L. incrustata* DAOM 239500 with black exudates; f. *L. incrustata* DAOM 239498; g. *L. incrustata* HNLHM91 with dried clear exudates; h. *L. incrustata* DAOM 239517; i. *Leohumicola* sp. DAOM 239516. — Scale bar = 5 mm.

grew slowly, and initially were yellow and slowly maturing to grey or olive colours, with brown or olive soluble pigments released into the surrounding media (Fig. 2, 3). Characteristic *Leohumicola* aleurioconidia and chlamydospores were observed for several strains after 3 mo on PDA and CMA, although some strains did not sporulate even after 6 mo in culture. *Leohumicola incrustata* DAOM 239517 only sporulated on PCA. The morphological descriptions of the new species *L. atra*, *L. incrustata*, and *L. levissima* are presented in the taxonomy section, and the characters of all known species are summarized in Table 2.

The internal transcribed spacer (ITS) nrDNA sequences were 462 bp long for all strains of *L. levissima*; 463 bp for most *L. incrustata* strains (except DAOM 239503, HNLHM91, HNLHM103, 464 bp, and DAOM 239500 462 bp) and 462 bp for both *L. atra* strains. ITS sequences of the isolated strains closely matched reference *Leohumicola* sequences in GenBank using BLAST searches. The barcode region of the cytochrome oxidase subunit I (*Cox1*) mitochondrial gene was 628 bp for all strains, except DAOM 239499 and DAOM 239516, which had an intron making the PCR product roughly 1500 bp. There are currently few fungal *Cox1* reference sequences in GenBank, but BLAST results of *Cox1* sequences from our strains matched fungal sequences for that gene at a maximum of 87 % DNA sequence identity. The sequences generated for *Leohumicola* and the outgroup *Myxotrichum deflexum* are currently the only *Leotiomycetes* with *Cox1* DNA barcode sequences.

Parsimony analyses of ITS and *Cox1* alignments (Fig. 4a, b) revealed that all previously recognized and newly discovered *Leohumicola* species form monophyletic groups in strict consensus trees, with weak bootstrap support for some clades. In the ITS analysis, *L. atra* (bootstrap support 87 %) and *L. incrustata* (98 %) form well-supported clades. *Leohumicola levissima* is paraphyletic with *L. minima* and two unidentified strains, but is resolved as a monophyletic group in the *Cox1* tree. In the *Cox1* analysis, the three new species are monophyletic, supported by bootstrap values of 73 % for *L. levissima*, 77 % for *L. atra*, and 99 % for *L. incrustata*. The monophyly of the strains of the type species of the genus *L. verrucosa* is not strongly supported by bootstrapping. The topology of the ITS and *Cox1* trees present differing sister group relationships. In the *Cox1*

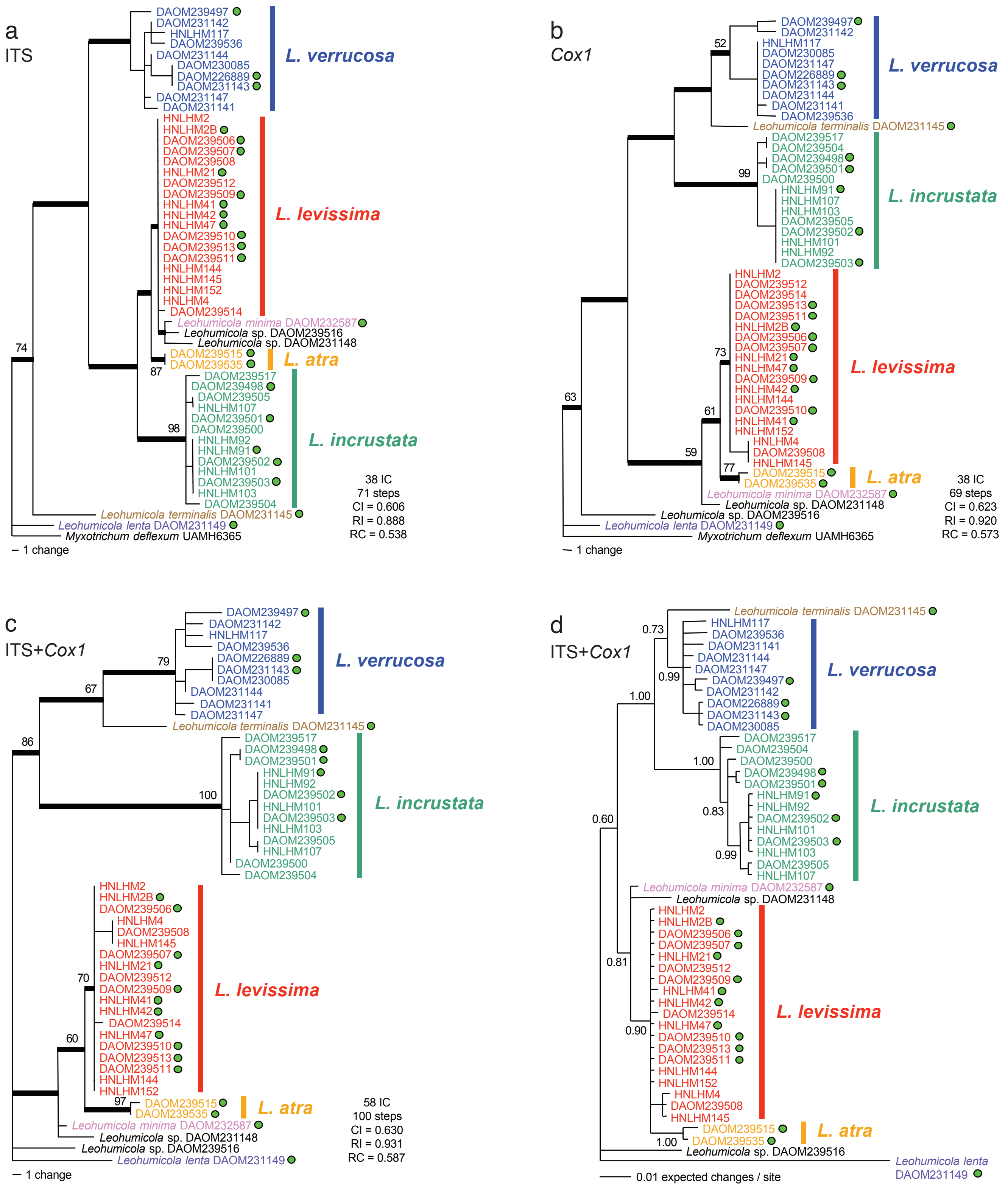


Fig. 4 ITS and *Cox1* phylogenetic analyses. a–c. Single most parsimonious trees based on heuristic analysis. Thick lines indicate branch topology retained in the strict consensus of the MPTs. Green dots represent strains that produced aleurioconidia and chlamydospores. Bootstrap support values above 50 % from 1 000 replicates of a full heuristic search for a and b, and from 1 000 replicates of a fast-step wise search for c are shown; d. Bayesian inference consensus tree based on 20 002 trees from a gene partition analysis of the combined ITS and *Cox1* data set. The K80+G and HKY+I models were used for the ITS partition and *Cox1* partition, respectively. Abbreviations: IC = informative characters, CI = consistency index, RI = retention index, RC = rescaled consistency index.

analysis, *L. terminalis* is sister to *L. verrucosa* whereas it is sister to *L. lenta* in the ITS analysis. Furthermore, *L. incrustata* is sister to the *L. atra/L. minima* clade in the ITS analysis, but most closely related to the *L. verrucosa/L. terminalis* clade in the *Cox1* analysis.

The partition homogeneity test confirmed that the ITS and *Cox1* data sets could be combined (P = 0.50). A dual-gene parsimony analysis (Fig. 4c) and a dual-gene Bayesian inference (Fig. 4d) were performed using *L. lenta* to root the tree.

In the dual-gene parsimony analysis, all three new species form monophyletic groups supported by strict consensus tree topology and bootstrap values of 70 % for *L. levissima*, 97 % for *L. atra*, and 100 % for *L. incrustata*. In the Bayesian analysis (Fig. 4d), *L. atra* and *L. incrustata* samples form monophyletic clusters both with a branch support value of 1.00. However, *L. levissima* is paraphyletic with *L. atra* in the Bayesian analysis. *Leohumicola verrucosa* is more strongly supported as a clade in both dual-gene analyses (79 % in parsimony and 0.99 in Bayesian) than in the single-gene analyses. The topology of the dual-gene parsimony and Bayesian analyses are identical and most similar to the *Cox1* parsimony analysis (Fig. 4b).

As reviewed by Seifert et al. (2007), introns are a frequently reported problem in fungal *Cox1* genes. Most of our strains of *Leohumicola* amplified easily but three strains were problematic. In DAOM 230084, chromatograms contained an ambiguous stretch with double peaks from position 574 to 590 of the *Cox1* amplicon; consequently, we removed this strain from all phylogenetic analyses. Both *Leohumicola* sp. DAOM 239516 and *Leohumicola* sp. DAOM 239499 had large introns (about 875 bp) in the target region of the *Cox1* gene. When these introns were removed and the sequences were translated, the amino acid alignment revealed conserved protein sequences in relation to the *Cox1* of most other *Leohumicola* strains. ITS parsimony analyses placed *Leohumicola* sp. DAOM 239499 (a sterile strain from South Africa) in the *L. incrustata* clade (data not shown), which corresponds with its colony characters. However, in a *Cox1* parsimony analysis with the intron

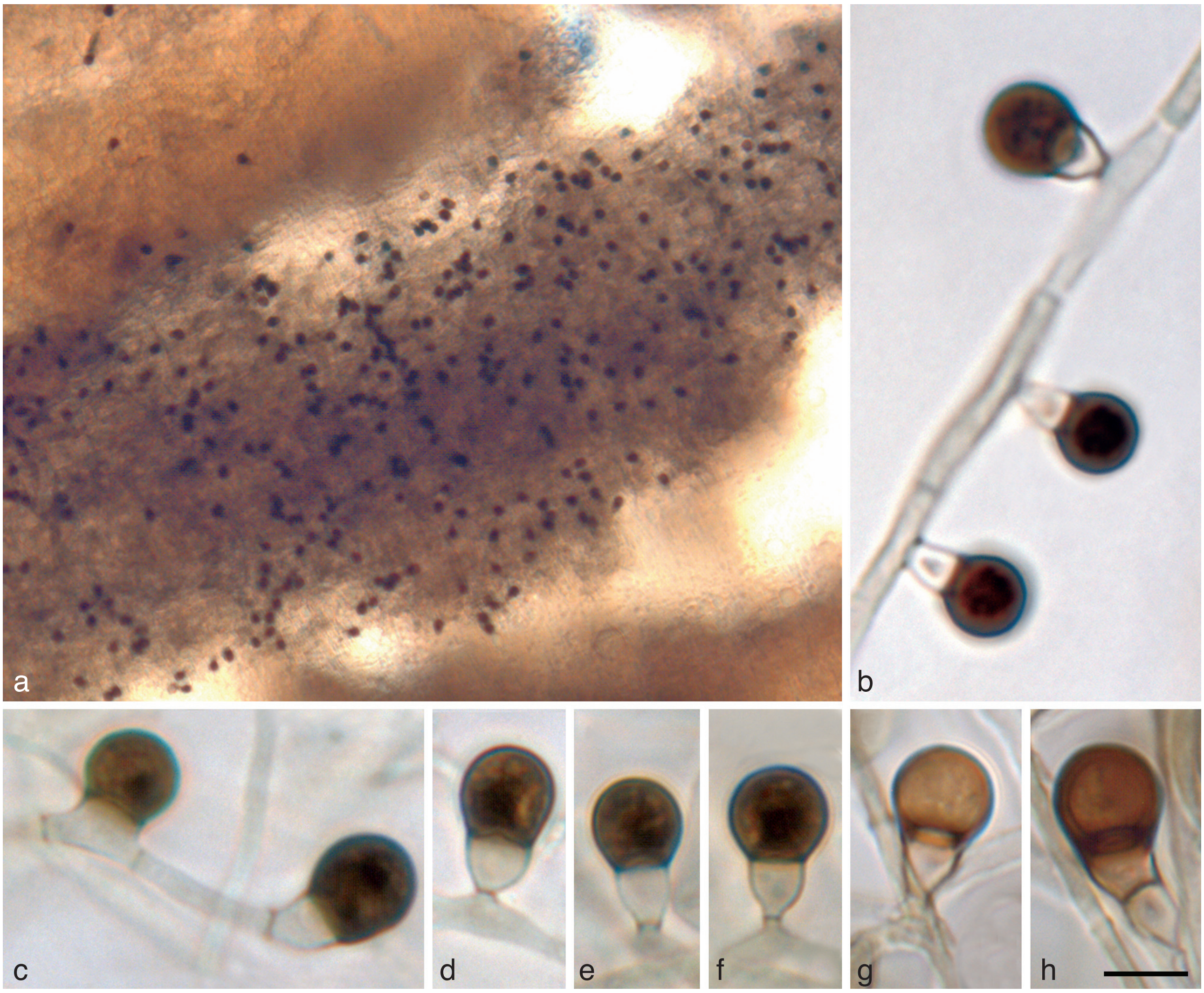


Fig. 5 *Leohumicola atra*. a. Sporulating part of colony on PDA; b. aleurioconidial development; c. single-celled aleurioconidium (left) and terminal aleurioconidium (right); d–f. mature aleurioconidia; g, h. lighter coloured aleurioconidia. All panels = DAOM 239515. — Scale bars: a = 50 μm, b–h = 5 μm.

removed, this strain formed a monophyletic clade with another intron-containing strain, *Leohumicola* sp. DAOM 239516 (from Ontario, Canada). The phylogenetic conflicts between ITS and *Cox1* were unexpected and thus strains DAOM 239499 and 239516 remain unidentified. Strain DAOM 239499 was removed from all analyses presented here to increase phylogenetic congruency, and allow our ITS and *Cox1* data to be combined for dual-gene analysis.

Taxonomy

Leohumicola atra Nguyen & Seifert, *sp. nov.* — MycoBank MB512015; Fig. 5

Conidia lateralia vel modice terminalia, cellula terminali 4.5–5.5 × 4.0–5.5 µm, (sub)globosa, atra, laevi; cellula basilari 2.5–4.5 × 2.5–3.5 µm, crateriformis, obconica vel cylindrica. Coloniae in agaro PDA dicto ca. 10 diam post 14 dies.

Holotypus. Cultura ex solo isolata, exsiccata in herbario DAOM 239515, viva ex-typo CCFC.

Etymology. Named after the dark-brown or nearly black colour of the terminal conidial cells (*atra* Lat. = dark).

Conidiogenous hyphae hyaline, approximately 1–2.5 µm wide, often in fascicles in aerial mycelium (Fig. 5a). *Conidiogenous cells* reduced to a single denticle 0.5–1.0 µm long (mean ± SE = 0.6 ± 0.1, n = 10) and 1.0–2.0 µm wide (1.5 ± 0.1, n = 10). *Conidia* initially two-celled, single or side by side in small clusters, or successively produced sympodially from hyphae, either lateral (Fig. 5b, d–f) or terminal on conidiogenous hyphae. Terminal cell 4.5–5.5 × 4.0–5.5 µm (5.0 ± 0.1 × 5.2 ± 0.1, n = 20), globose to subglobose, at first hyaline like the basal cell, becoming dark brown (Fig. 5h) or almost black; conidial walls slightly thick, remaining smooth after 3 mo. Conidial connection to basal cell 2.5–3.5 µm wide, not constricted. Aleurioconidia sometimes single-celled with terminal cell directly attached to the hypha, with no basal cell (Fig. 5c). Basal cell 2.5–4.5 × 2.5–3.5 µm (3.3 ± 0.1 × 3.1 ± 0.1, n = 20), obconical or cupulate, often symmetrical or sometimes asymmetrical or irregular, hyaline or slightly pale brown, paler than the terminal cell. Ratio of lengths of terminal : basal cell 1.5–2.0 (1.6 ± 0.1). Basal cell of conidium rupturing during secession, resulting in a functionally single-celled conidium bearing the remnant of the basal cell. *Chlamydospores* sparsely produced, intercalary, single, rarely in chains, concolorous with conidial terminal cell, subglobose to ellipsoidal, 3–11 × 4–7 µm, with thin or slightly thickened walls. *Vegetative mycelium* often with swollen, monilioid, hyaline or subhyaline hyphae 1.5–2 µm wide, septate, with slightly thickened walls.

Colonies on PDA after 2 wk (Fig. 2) under ambient light at room temperature roughly 10 mm diam; entirely olive (3E3) at first becoming grey (3E1) in the centre and olive (3E3) at the margins as the colonies mature, convex, wrinkled with a felty appearance. Exudates produced around the colony centre as black droplets; soluble pigments greyish red (8C4) or faint yellow/orange around the colony and becoming darker with age. Margin entire, slightly gnawed, or smooth. Colony reverse dark-brown (7F8).

Colonies on OA after 2 wk (Fig. 2) under ambient light at room temperature roughly 13–15 mm diam; grey (4E1) in the centre and olive-brown (4E4) at the margins, sometimes with felty white aerial mycelium; soluble pigments brown (6E4) around the colony. Margin entire and smooth. Colony reverse brown (5F5) to olive (3F5).

Specimens examined. USA, Oregon, Crater Lake National Park, 45°56'N 122°08'W, from heated soil, 28 July 2006, *M. Trappe*, holotype and ex-type strain DAOM 239515. Two strains were isolated from one soil sample (Table 1).

Notes — The terminal cell of the conidia of *L. atra* becomes much darker brown compared to other *Leohumicola* species and is nearly black. It remains smooth-walled even after 5 mo incubation.

Leohumicola incrustata Nguyen & Seifert, *sp. nov.* — MycoBank MB512014; Fig. 6

Conidia lateralia vel modice terminalia, cellula terminali 4.0–5.5 × 4.0–5.0 µm, (sub)globosa, brunnea, incrustata; cellula basilari 2.5–4.5 × 2.0–3.0 µm, crateriformis vel obconica. Coloniae in agaro PDA dicto 12–18 mm diam post 14 dies.

Holotypus. Cultura ex solo isolata, exsiccata in herbario DAOM 239498, viva ex-typo CCFC.

Etymology. Named after the appearance of the terminal conidial cells which are incrusted with a crust-like slime and warts.

Conidiogenous hyphae hyaline, 1.5–2.0 µm wide, often in fascicles in aerial mycelium. *Conidiogenous cells* reduced to a single denticle 1.0–3.0 µm long (mean ± SE = 1.4 ± 0.1, n = 15) and 1.5–3.5 µm wide (2.1 ± 0.1, n = 15). *Conidia* initially two-celled, single or side by side in small clusters, or successively produced sympodially (Fig. 6a, e) from hyphae, either lateral or terminal on conidiogenous hyphae. Terminal cell 4.0–5.5 × 4.0–5.0 µm (4.9 ± 0.1 × 4.5 ± 0.1, n = 20), globose to subglobose, at first hyaline like the basal cell, becoming either pale brown to dark brown; conidial walls slightly thick, smooth or slightly verrucose with large warts 0.75–1.5 µm, usually incrusted with a brown coloured slime 1–2 µm thick around the apex (Fig. 6b–d, f–l). Conidial connection to basal cell 2–3 µm wide, not constricted. Basal cell 2.5–4.5 × 2.0–3.0 µm (3.7 ± 0.1 × 2.7 ± 0.1, n = 20), obconical or cupulate, often symmetrical or sometimes asymmetrical or irregular, hyaline to pale brown, paler than the terminal cell. Ratio of lengths of terminal : basal cell 1.0–2.0 (1.5 ± 0.1). Basal cell of conidium rupturing during secession, resulting in a functionally single-celled conidium bearing the remnant of the basal cell. *Chlamydospores* sparsely produced in submerged mycelium, commonly found in poorly sporulating colonies, intercalary, single, concolorous with conidial terminal cell, subglobose or ellipsoidal or irregularly shaped with a rough and wrinkled appearance, 5–6 × 3–4.5 µm, with slightly thickened walls. *Vegetative mycelium* often with swollen, monilioid, hyaline or brown coloured hyphae 1.5–3 µm wide, septate, with slightly thickened walls.

Colonies on PDA after 2 wk (Fig. 2) under ambient light at room temperature 12–18 mm diam; pastel yellow (2A4) or greyish yellow (2B3) or grey (2B1) in the centre and olive-yellow (2C8) or white at the margins; sometimes wrinkled, sometimes splitting the agar near the colony centre, with short and felty white aerial mycelium. Exudates produced; around colony centre

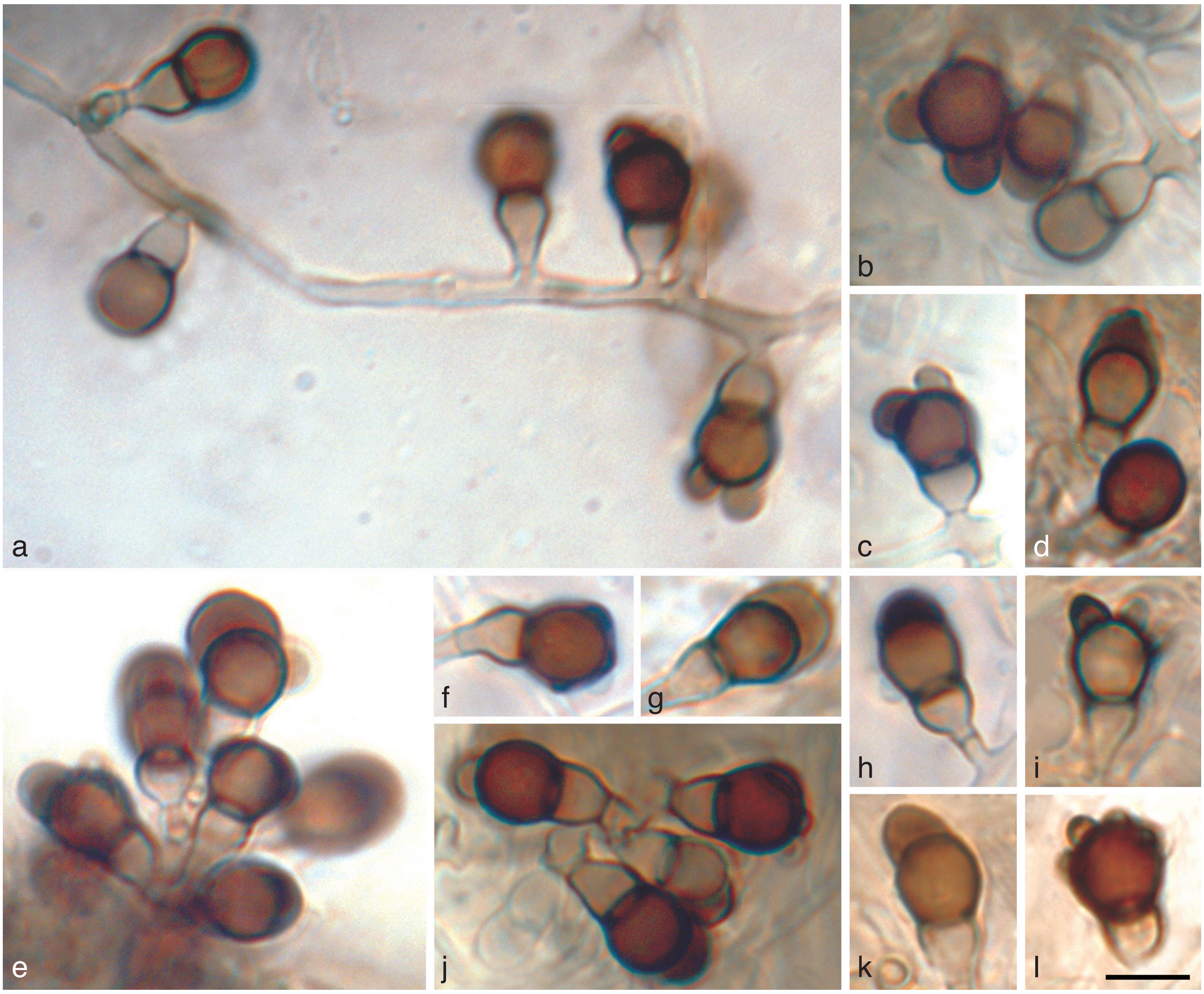


Fig. 6 *Leohumicola incrustata*. a, e. Aleurioconidial development; b–d, f–l. terminal cells of aleurioconidia are often incrusted with a brown slime or warts around the apex. Panels a–c, e, j = DAOM 239501; d, f–i, k, l = DAOM239502. The background of panel j was altered digitally for aesthetic reasons. — Scale bar = 5 µm.

as small reddish brown droplets, at the colony margins and centre as large clear droplets, or around the colony centre as small dark brown to black droplets; soluble pigments variable ranging from brown (6D8), olive (3E8), and dark yellow (4C8). Margin smooth, entire, sometimes irregular or gnawed. Colony reverse dark-olive (2F6) or dark-brown (5F8).

Colonies on OA after 2 wk (Fig. 2) under ambient light at room temperature roughly 15–20 mm diam; yellowish white (2A2) to pastel-yellow (2A4); soluble pigments sometimes absent and sometimes minimal and purplish white (14A2) around the colony. Margin entire and smooth. Colony reverse pale yellow (3A2) to light yellow (3A5) at the margin and olive (2E3) in the centre.

Specimens examined. South Africa, Western Cape Province, Cape of Good Hope Nature Reserve, 34°20'S 18°27'E, from heated soil, 7 April 2006, *K.A. Seifert*, holotype and ex-type strain DAOM 239498. Thirteen strains were isolated from four soil samples (Table 1).

Notes — Large warts or slime production on the terminal cell are the defining characteristics of *L. incrustata* aleurioconidia. The conidia are most similar to those of *L. verrucosa*, but in that species the wall ornamentation forms smaller, discrete warts.

Leohumicola levissima Nguyen & Seifert, *sp. nov.* — MycoBank MB512013; Fig. 7

Conidia lateralia vel modice terminalia, cellula terminali 4.5–6.0 × 4.0–5.5 µm, (sub)globosa, brunnea, laevi; cellula basilari 1.5–4.0 × 2.5–3.5 µm, crateriformis vel obconica. Coloniae in agaro PDA dicto 15–20 mm diam post 14 dies.

Holotypus. Cultura ex solo isolata, exsiccata in herbario DAOM 239506, viva ex-typo CCFC.

Etymology. Named after the smooth walled and unornamented appearance of the terminal conidial cells (*levissima* Lat. = smooth).

Conidiogenous hyphae hyaline, 1–2 µm wide, often in fascicles in aerial mycelium. *Conidiogenous cells* reduced to a single denticle, 0.5–1.5 µm long (mean ± SE = 0.8 ± 0.1, n = 12) and 1.0–3.5 µm wide (2.2 ± 0.2, n = 12). *Conidia* initially two-celled, single (Fig. 7j) or side by side in small clusters, or successively produced sympodially (Fig. 7a) from hyphae, either lateral (Fig. 7b) or terminal (Fig. 7c) on conidiogenous hyphae. Terminal cell 4.5–6.0 × 4.0–5.5 µm (5.3 ± 0.1 × 4.9 ± 0.1, n = 20), globose to subglobose, at first the same colour as the basal cell, becoming dark brown while still attached; conidial walls slightly thick, remaining smooth after 3 mo. Conidial connection to basal cell 2.5–3.5 µm wide, not constricted. Basal cell 1.5–4.0 × 2.5–3.5 µm (2.6 ± 0.1 × 3.1 ± 0.1, n = 20), obconical or cupulate, often symmetrical or sometimes asymmetrical or irregular, hyaline to pale brown, paler than the terminal cell. Ratio of lengths of terminal : basal cell 1.5–2.5 (1.8 ± 0.1). Basal cell of conidium rupturing during secession (Fig. 7e), resulting in a functionally single-celled conidium bearing the remnant of the basal cell (Fig. 7k). *Chlamydospores* sparsely produced in submerged mycelium, commonly found in poorly sporulating colonies, intercalary, single, concolorous with conidial terminal cell, subglobose to ellipsoidal, sometimes with irregular constrictions, 5.5–7.5 × 5–6 µm, with thin or slightly thickened walls (Fig. 7f–i). *Vegetative mycelium* often with swollen, monilioid, hyaline or subhyaline hyphae 1–2 µm wide, septate, with slightly thickened walls.

Colonies on PDA after 2 wk (Fig. 2) under ambient light at room temperature 15–20 mm diam; olive (2E4) or grey (2D1) in the centre and greyish yellow (2C4) or yellowish grey (2C2) at the margins, planar or convex, sometimes wrinkled, sometimes splitting the agar near the colony centre, with low, felty, slightly lanose white aerial mycelium. Soluble pigments not produced after 2 wk. Margin smooth and entire. Colony reverse olive-grey (2F2) to olive (2E4).

Colonies on OA after 2 wk (Fig. 2) under ambient light at room temperature roughly 12–17 mm diam; pale yellow (2A3) to light yellow (2A5); soluble pigments minimal around the colony. Margin entire and smooth. Colony reverse olive (2E5) in the centre and light yellow (3A5) at the edge.

Specimens examined. USA, Oregon, Crater Lake National Park, 45°56'N 122°08'W, from heated soil, 28 July 2006, *M. Trappe*, holotype and ex-type culture DAOM 239506. Nineteen strains were isolated from three soil samples (Table 1).

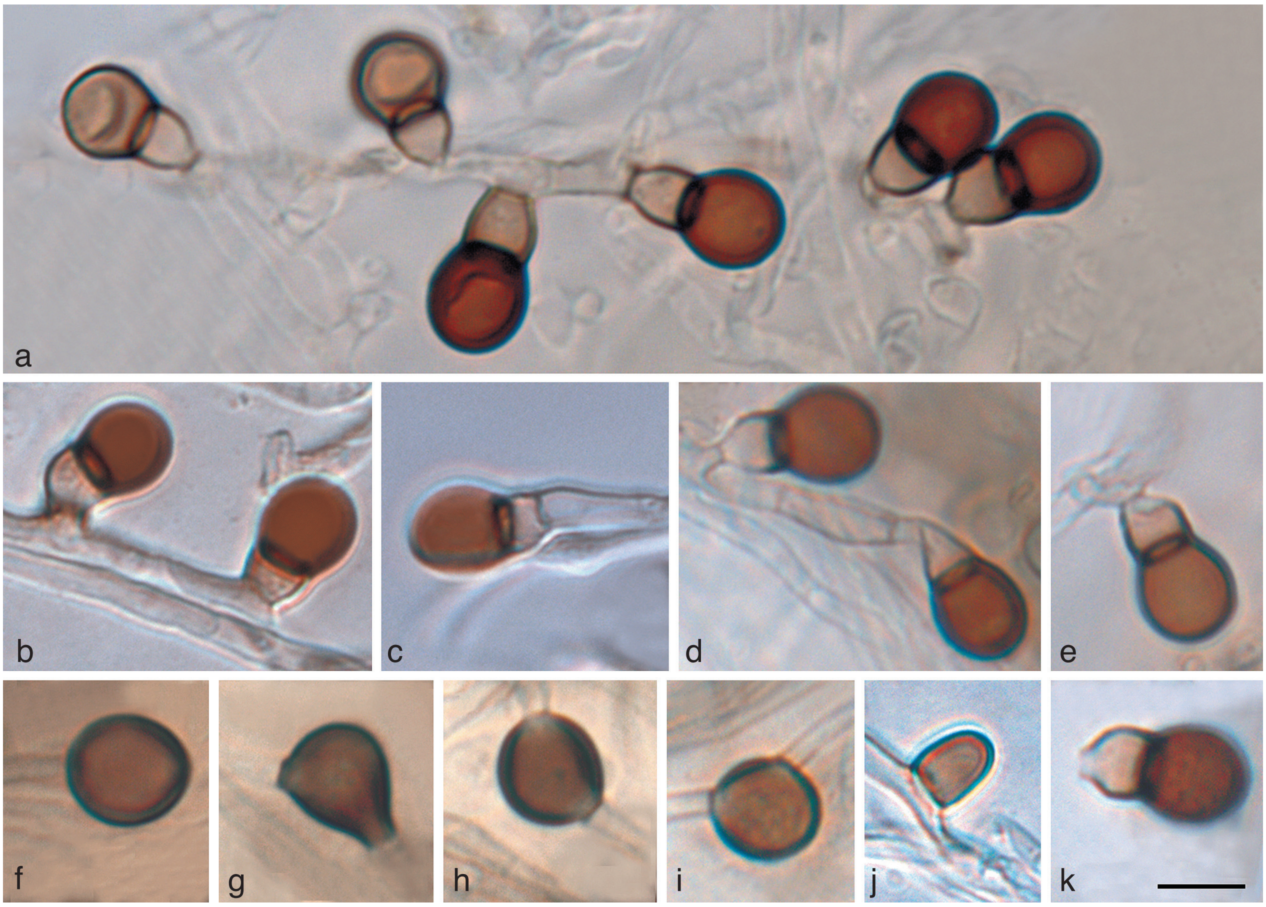


Fig. 7 *Leohumicola levissima*. a. Aleurioconidial development, with younger aleurioconidia (left) and older aleurioconidia (right); b. laterial aleurioconidia; c. terminal aleurioconidium; d. laterial (left) and terminal (right) aleurioconidia; e. basal cell of aleurioconidium rupturing during secession; f. terminal chlamydospore; g–i. intercalary chlamydospores; j. single-celled aleurioconidium; k. functionally single-celled aleurioconidum bearing the remnant of the basal cell. Panels a, d, e, k = DAOM239511; b, c, j = DAOM 239509; f–i = DAOM 239513. The background of panels a and c were altered digitally for aesthetic reasons. — Scale bar = 5 µm.

Table 3 Comparison of ITS and *Cox1* markers for DNA barcoding.

Marker	No. of isolates analysed	No. of species	Sequence length	Mean infraspecific divergence (%)	Range of means (%)	Mean interspecific divergence (%)	Range of means (%)
ITS	45	7	462	0.35	0.04–0.78	3.71	0.45–8.09
Cox1	45	7	628	0.24	0.11–0.32	2.42	0.54–4.99

Notes — The terminal cell of *L. levissima* conidia remains smooth even after 3 mo, in contrast to the roughened or encrusted terminal cells of *L. verrucosa* and *L. incrustata*. The conidia of *L. atra*, described above, have similarly smooth terminal cells, but are much darker. The colony morphologies of *L. levissima* and *L. atra* are distinct, particularly on OA. On OA, *L. levissima* is light yellow and *L. atra* is grey and olive brown (Fig. 2). Also, *L. levissima* grows faster on PDA than *L. atra*. Based on the similarity in aleurioconidia and the topology of the parsimony analyses, perhaps these two species share a common ancestor. PDA colonies of *L. levissima* after 6 wk incubation are less variable than they are after 2 wk. Older colonies eventually produce pale olive, soluble pigments.

DISCUSSION

This study proposes three new species of *Leohumicola*, namely *L. atra*, *L. incrustata*, and *L. levissima*, using morphological characters and DNA barcoding. The three species exhibit the characteristic two-celled aleurioconidia of the genus, which become functionally single-celled after secession. Brown to olive pigments diffuse into agar media, and colonies grow slowly. The new species can be distinguished by features of the terminal cells of the aleurioconidia. In *L. levissima* they are smooth and brown, whereas they are smooth and almost black in *L. atra*, and covered with warts (that sometimes appear slimy rather than composed of cell wall material) in *L. incrustata*. Chlamydospores, similar in pigmentation to the aleurioconidia, are produced by the three new species and the four previously described species. A revised key to the seven known species of *Leohumicola* is provided below.

Dense mycelial growth, and abundant soluble pigment production on PDA, renders microscopic observations of *Leohumicola* conidia difficult. They are more conspicuous on the optically clear CMA medium, where the mycelia are sparser and soluble pigments are reduced; sporulation is more abundant on inoculum blocks originally transferred from PDA. Generally, strains were more likely to produce conidia after 3 mo on CMA than on PDA, OA, or when macerated on PDA. As indicated on the phylogenetic trees, not all strains of *L. incrustata* and *L. levissima* sporulated. Of note, *Leohumicola* sp. DAOM 239516 produced almost exclusively chlamydospores and only one aleurioconidium was seen. This isolate is phylogenetically distinct from other *Leohumicola* species, but we chose not to describe it here because of the paucity of diagnostic morphological characters in the single culture available.

The results of our phylogenetic analyses are in agreement with the morphological data. Most *Leohumicola* species are monophyletic, although *L. levissima* is paraphyletic with *L. minima* in the ITS parsimony analysis and with *L. atra* in the dual-gene Bayesian analysis. Bootstrap support is variable for some groups and the sister relationships among species are inconsistent. Although this renders the phylogenetic structure of the genus uncertain, it does not interfere with monophyletic species recognition. Perhaps as additional species are discovered and the species sampling of this genus is more complete, the phylogenetic structure will be clearer.

As DNA barcodes, the ITS and *Cox1* loci provide similar sequence variation and reveal similar phylogenetic groupings of *Leohumicola* species (Table 3). However, *Cox1* provides slightly better species resolution, particularly for the *minima/levissima/atra* clade. Both *Cox1* and ITS sequences are suitable DNA barcodes for the currently known *Leohumicola* species because the mean sequence divergence between species is about 10 times greater than the mean divergence within species.

Genetic anomalies in the target region of the *Cox1* gene were found in three *Leohumicola* isolates. *Leohumicola* sp. DAOM 230084 contained a short ambiguous stretch of double peaks that may indicate multiple copies of the *Cox1* gene. *Leohumicola* sp. DAOM 239499 and DAOM 239516 contained introns. They were partially sequenced from the 5' and 3' ends, but additional sequencing primers would be needed to sequence the entire length of the introns.

The search for *Leohumicola* species has only begun. Hambleton et al. (2005) noted that *Leohumicola* ITS sequences correspond (with > 95 % identity and BLAST E-value of 0) to many unidentified fungi from environmental studies or plant roots in GenBank. Based on these observations and our discovery of three new species from soil samples from two previously unsampled locations, *Leohumicola* is likely to be a diverse, wide-spread soil-borne genus. The soil heating procedure should be effective for isolating additional *Leohumicola* species from other countries.

KEY TO THE SPECIES OF *LEOHUMICOLA*

1. Colony diam < 5 mm after 2 wk on PDA, terminal cell of conidium 7–10 μm long . . . . *L. lenta*
1. Colony diam > 5 mm after 2 wk on PDA, terminal cell of conidium shorter 2
2. Conidia lateral and terminal 3
2. Conidia terminal only and usually smooth-walled . . . . *L. terminalis*
3. Terminal cell of conidium mostly ellipsoidal . . . . *L. minima*
3. Terminal cell of conidium globose or subglobose, with a width of about 5 μm diam 4
4. Terminal cell of conidium smooth after 3 mo incubation . 5
4. Terminal cell of conidium verrucose or encrusted 6

5. Terminal cell of conidium pale brown .... *L. levissima*
5. Terminal cell of conidium dark-brown .... *L. atra*

6. Many small warts occurring on the terminal cell of the conidium .... *L. verrucosa*
6. Few large warts or slime produced around the apex of the terminal cell .... *L. incrustata*

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